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TITLE: Oncogenic LINE-1 Retroelements Sustain Prostate Tumor Cells and Promote Metastatic Progression

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this hypothesis development project is (1) to determine if ectopic expression of LINE-1 elements in prostate cancer contribute to its progression by activating oncogenic DNA sequences, or silencing tumor suppressor like sequences. We have RNA-sequencing data that we are part way through processing, but suggests so far significant activation of non-coding RNA sequences derived from RNA from a lymph node metastasis from the prostate. Furthermore, we have subcloned a LINE-1 Open reading frame sequence and will determine the effect of its expression in non-tumorigenic prostate cells. Finally, we have cloned the PIWIL-1 gene, known as a repressor of LINE-1 retroelement sequences in the testis, and have it under the control of a doxycycline-inducible promoter in a lentiviral system, and expressed this in LNCaP and PC-3 prostate cancer cells. Experiments are ongoing to determine if PIWIL-1 expression in prostate cancer cells will reduce their growth, thereby providing proof of principle for future gene-based therapeutics for this cancer.

15. SUBJECT TERMS
Prostate cancer, LINE-1, PIWIL-1, retrotransposons

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

We proposed that ectopic expression of LINE-1 in prostate cancer is oncogenic and leads to *advanced* prostate cancer progression, by activating novel oncogenic transcriptional pathways and by acting as a telomerase thereby contributing to immortalization of the metastases. We also proposed that expression of the retrotransposon-repressing PIWI protein in these cells will block LINE-1 transcriptional activation and therefore growth of prostate cancer metastases, thereby providing proof-of-principle for a novel therapeutic target present in virtually all prostate cancer metastases.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

PIWIL1 retrotransposon LINE-1 telomerase prostate

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The project was divided into three main tasks; for Task 1, the goal was to determine whether expression of LINE-1 encoded proteins can transform non-tumorigenic prostate cells and whether LINE-1 ORF expression confers a metastatic phenotype. For Task 2, the plan was to determine the effect active LINE-1 transcription has on gene expression in metastatic prca. And for Task 3, we planned to determine if LINE-1 expression is essential for maintenance and progression of metastatic disease and provide “proof of principle” for a potential therapy targeted at LINE-1 expression in advanced prostate cancer. Task 4 is writing up the results and presenting at a conference.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

The project was divided into three main tasks, and regulatory approvals were required for each of the three tasks, initially from UTHSCSA and then from the CDMRP for the use of human tissues and cell lines, the use of animals and the use of recombinant DNA/lentiviral vectors. All of these approvals have now been obtained. For Task 1, we cloned the LINE-1 Open Reading Frame sequences, ORF1 and 2, using standard PCR cloning techniques. However it is not clear if these particular clones are active in terms of producing a telomerase as there are many different mutant variants of LINE-1 in human tumors. For Task 2, we identified Lymph node metastasis tumor tissues in the UTHSCSA tissue bank, however the RNA was not considered of sufficient quality to submit for RNA sequencing. We did RNA sequencing of LNCaP cell line RNA as this is derived from a prostate cancer lymph node metastatic deposit, although the bioinformatics analysis has proved challenging and we are mid-way. For Task 3, we cloned the main variant of PIWIL1, and have it under tetracycline/doxycycline control in a lentiviral vector. We used this to generate stable cell lines that we are characterizing and implanting mice.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report – the project was not intended to provide a training opportunity

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

While training was not a primary goal of this project, a graduate student in the lab has worked one-on-one beside myself while developing the constructs and cell lines for the project. Therefore the project has served to increase the professional development of the student in the areas of prostate cancer research and molecular biology techniques (cloning, sequence analysis, lentiviral construction and cell transduction, gene expression, western blotting and protein expression).

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Initially we are focused on completing Task 3, by characterizing our novel cell lines expressing PIWIL1, and determining if tumor xenografts in mice established from these cell lines can provide proof-of-principle that expression of PIWIL1 can act as a tumor toxin and block LINE1 expression, and therefore stop prostate cancer progression. Additionally, we plan to continue our bioinformatics analysis of RNA-seq data to isolate LINE-1 containing transcripts, and expression of LINE1 ORF 1 and 2 in non tumorigenic lines to see if they become transformed (Tasks 1 and 2).

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The generation of cell lines with inducible-PIWIL1 expression will be of use to others studying the role of this gene and the associated pathway. Currently, there is confusion in the literature as to whether PIWIL1 is in fact pro- or anti- oncogenic. Our study and cell lines will help answer this question correctly.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The inducible PIWIL1 construct will be useful to people studying fields other than cancer, for example embryogenesis and reproduction, as PIWIL1 is important in these processes.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*

- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

There was significant problems with the project in that the RNA quality from the sample specimens (prostate cancer metastases) was so low that it could not be used for RNA sequencing. We are resolving this problem by making sure that the pathology department and the surgeons operating are aware that metastatic deposits need to be immediately frozen if they are to be banked in the tissue bank for future use. This seems to be proving to be successful. Additionally, the project was held up because some of the approaches (lentiviral transduction) are apparently not amenable to expression of viral-type sequences (the LINE-1 ORFs). However, while a 12 month extension of the project was requested and granted, there has been not significant changes in the project or its direction.

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Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

As mentioned above, a significant problem has been the acquisition of freshly banked metastatic lymph node tissue that could yield RNA with acceptable "RIN"s (RNA integrity numbers), so that they could be analyzed by RNA-sequencing for Major Task 2. We are resolving the problem by 1) sequencing LNCaP RNA (a cell line derived from a prostate cancer metastasis) and by implementing awareness of the project with our clinicians who are currently operating on patients with metastasis, so that the tissue can be immediately frozen and banked. While most patients don't have metastatic deposits discovered during surgery, we routinely have cases that do have lymph node metastases, and the tissue bank is now consenting all patients presenting for prostatectomy so that if metastasis is recognized during surgery it will be collected. Additional problems include demonstrating PIWIL1 protein presence in the induced cultures. There are very few antibodies available that detect PIWIL1 – while we can detect mouse PIWIL1, we have been unable to detect human PIWIL1 despite robust (200+x) induction of the gene in our induced cell lines (see attached figures).

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

There were delays in hiring staff, as we did not want to do this until all regulatory approvals were through. As such we have requested and received a no cost extension for this project in order to complete it.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

There was no significant deviations or unexpected outcomes regarding the use or care of human subjects.

There was no significant deviations or unexpected outcomes regarding the use or care of vertebrate animals.

Significant changes in use of biohazards and/or select agents

There was no significant change in the use of biohazards and/or select agents.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

We have produced vectors containing PIWIL1 expressed under the control of a tetracycline inducible promoter and cloning vectors containing LINE1 ORF1 and 2.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

PI Denise S O'Keefe
Co-I Dean J Bacich
No change [the graduate student participated for less than one calendar month]

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Appendix
Supporting Data for Reporting Period

Cloning strategy for Inducible PIWIL1 expression

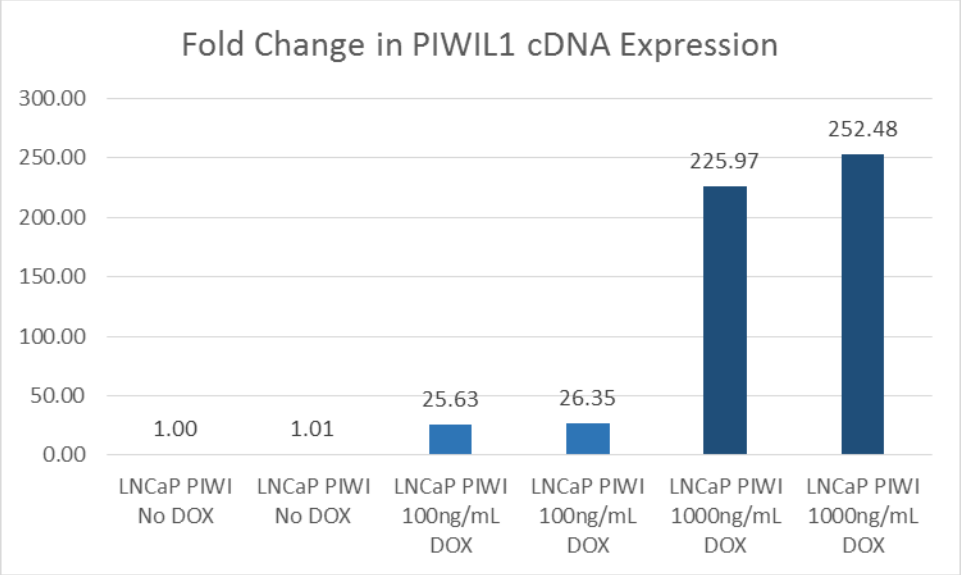
1. A TrueORF clone for PIWIL1 (NM_004764) was ordered from Origene, in the pCMV6 vector. This vector can be used in transfections to produce approximately 95kD encoded protein. If PIWIL1, as we propose is actually a cancer cell toxin, transfection with PIWIL1 would result in prostate cancer cell death with no way to study if the death is due to PIWIL1 expression or the mode of transfection. We therefore undertook cloning of PIWIL1 into a tetracycline inducible vector, pLVX-TET-ONE puro (Takara Clontech).

The cloning primers were:
F PIWIL1 cloning
5' CCCTCGTAAAGAATT-CATGACTGGGAGAGCCCGAGCCAGA 3'
R PIWIL1 cloning
5' GAGGTGGTCTGGATCCTTA-GAGGTAGTAAAGGCGGTTTGA 3'

Several clones were sequenced and clone 5 was chosen for lentiviral production and transduction of cell lines.

LNCaP and PC3 cells were transduced and stable lines were selected for using puromycin.

Figure 1: Transcriptional induction of PIWIL1 in LnCaP cells using the tetracycline analog, doxycycline.



However Western Blotting on protein lysates from the induced cells did not show PIWIL1 expression.

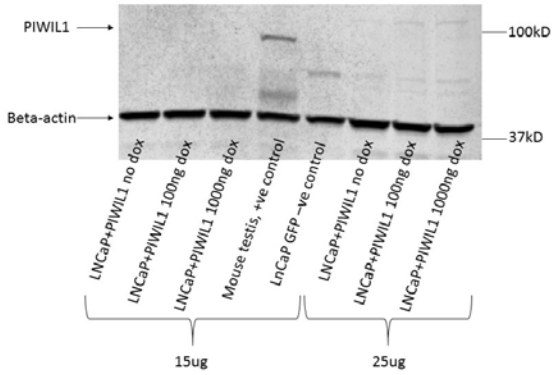
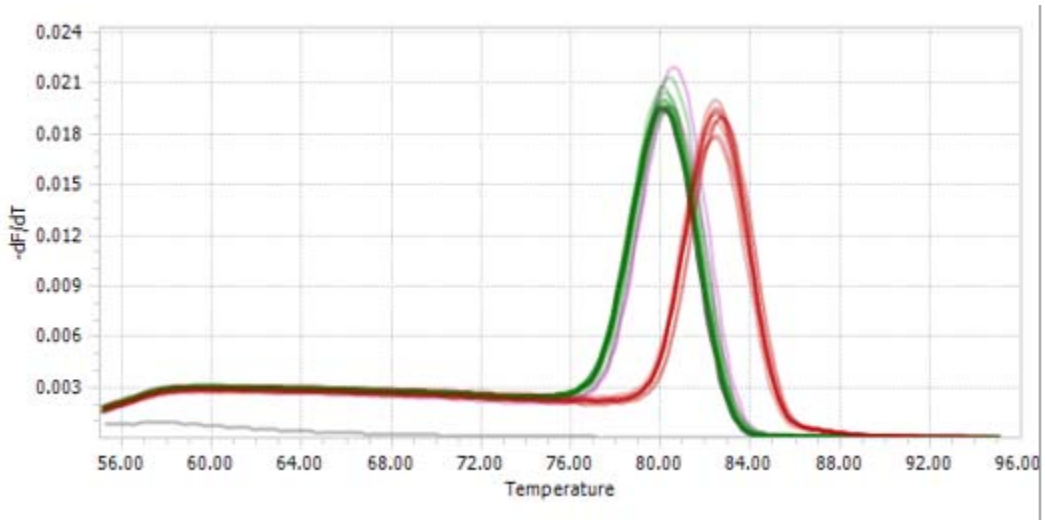


Figure 2: PIWIL1 protein expression determined via Western Blot with antibody PA5-19457 (Invitrogen). No expression of human PIWI is seen, raising the question of whether our induction of expression is within the range seen in mouse testis. We tested this using a quantitative PCR assay that targets both mouse and human PIWI (see Figure 3).

Figure 3: High resolution melt analysis of PIWIL1 RNA expression in mouse testis (green) and LNCaP cells showing induced expression of PIWIL1 (red). Despite nearly 100% identity, the two species PIWIL1 RNA is able to be distinguished by melting temperature. Additionally, quantitative analysis reveals nearly identical threshold cycles (data not shown), indicating very similar levels of PIWIL1 RNA in LNCaP induced cells and mouse testis.



These data indicate that the PIWIL1 antibody is not detecting human PIWIL1, despite being raised against a peptide derived from the human sequence. The company that supplied the antibody was contacted and they provided a different antibody that has been tested in humans, and the data obtained from that antibody will be included in the final report for this grant.